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Ethanol Fermentation on Mixed Sugars Using Mixed Culture of Two Yeast Strains

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Abstract

The objective of this study was to evaluate the use of mixed cultures of the recommended yeast strains from a previous study on ethanol fermentation using a substrate mixture consisting of sucrose, glucose, and fructose. There were three mixed (combination) cultures namely OUT7096/OUT7913, OUT7096/OUT7921, and OUT7913/OUT7921. The fermentation medium contained sugar mixture consisting of glucose, fructose, and sucrose with a composition generally close to the composition of sugars in sweet sorghum juice. Overall, fermentation is carried out in two stages. First fermentation was performed using the three mixed cultures to determine the best combination based on the concentration of ethanol produced and the concentration of residual sugar. Second fermentation was conducted using the best mixed culture obtained from the first stage. This second stage was intended to describe the pattern of the changes in the concentration of ethanol, sugars and biomass during the fermentation progresses and to determine some kinetic parameters namely ethanol yield ($Y_{p/s}$), growth yield ($Y_{x/s}$) and specific growth rate (μ). The results of the first fermentation showed that the best mixed culture was OUT7913/OUT7921 and the subsequent fermentation using this culture provide the highest ethanol yield ($Y_{p/s}$) = 0.47 $g.g^{-1}$ that was reached at 53rd hour, growth yield ($Y_{x/s}$) = 0.425 g.g-1, and μ = 0.12 $hour^{-1}$.

Keywords : fermentation, ethanol, mixed culture, mixed sugar

Introduction

Saccharomyces cerevisiae is widely used for the production of alcohol and ethanol fuel through a fermentation process that converting sugar into ethanol. This microbe can produce a lot of ethanol and has a high tolerance to ethanol and other growth inhibitors compounds (Balat *et al*., 2008). *S. cerevisiae* is able to produce 50 mmol of ethanol per hour per gram of cell protein in optimum conditions (Dombek and Ingram, 1987). Conversion of sugar into ethanol by *S. cerevisiae* takes place in anaerobic condition through a series of biochemical reactions called Embden Meyerhof Parnas pathway (EMP) or glycolysis which produces pyruvic acid. Furthermore, the pyruvic acid is converted to ethanol in two steps namely *decarboxylation* of pyruvic acid into acetaldehyde and then the reduction of the acetaldehyde to the ethanol (Lehninger *et al*., 2004).

The ethanol fermentation of substrates having a high concentration of sugar consisting of glucose, fructose, and sucrose could not fully proceed. This is because most of yeast is unable to convert sugar, especially fructose, completely during the fermentation (Wu *et al*., 2010). This problem occurs because the yeast prefers both sucrose and glucose to fructose. The fructose conversion takes place when the concentration of ethanol is already high enough to poison the yeast resulting

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in the low conversion of fructose (Wu *et al*., 2010). To enhance the conversion of the substrate, it is required a culture of yeast, which is not only has a high ability to convert glucose and sucrose, but also to convert the other sugar components including fructose.

Sweet sorghum is a very potential crop for bioethanol production because of some advantages over the other crops (Reddy *et al*., 2006). Sugar content in sweet sorghum juice is mainly consisted of sucrose, glucose, and fructose. Composition of the sugars depend on the variety, planting time (Teetor *et al*., 2010), and harvest time (Almodares *et al*., 2007). Generally, concentration of sucrose is the highest among the sugars concentration, where as glucose and fructose almost have the same concentration. Therefore, in order to use the juice in ethanol production, it is required a culture being able to convert all of the sugars into ethanol completely.

A certain yeast strain will has different capabilities when used on substrates of different sugars. Meanwhile, some substrates containing the same type of sugar with the same concentration would result in a different amount of ethanol when fermented with different yeast strains. Different results will also be obtained when the used sugar is in a mixed form (Jasman *et al*., 2012). Therefore, medium consisting of several different sugars may be more suitable fermented by mixed culture.

According to Hesseltine (1992), a mixed culture has the advantages over a single one. For example: (1) The yield of fermentation could be higher; (2) The rate of cell growth could be higher if one organism produces growth factors or compounds essential to the growth of other organisms; (3) Compounds made by a mixture of microorganisms often complement each other and work to the exclusion of unwanted microorganisms; (4) Mixed cultures permit better utilization of the substrate; (5) Mixed-culture fermentations enable the utilization of cheap and impure substrates.

The use of mixed culture in fermentation have been conducted in many cases but there has not been any report about using a mixed culture of two *Saccharomyces* strains in ethanol production from sugar mixture. Such cultures were used in this study to ethanol production from sugars mixture consisting of glucose, fructose, and sucrose with a composition generally close to that of sweet sorghum juice. This was performed to evaluate the capability of the cultures previous to applying it in ethanol production from sweet sorghum juice.

Materials and Method

Yeast

There are three recommended strains of yeasts from our previous study namely OUT7096, OUT7913, and OUT7921. The three strains of yeast were obtained from Prof. Satoshi Harashima, Dept. of Biotechnology, Fac. of Engineering, Osaka University, Japan. All yeast isolates were maintained on Malt Extract Agar (MEA) at 4°C in the refrigerator and were sub-cultured every 2 months.

Fermentation Media

Fermentation media were consisting of glucose, fructose, sucrose, yeast extract, peptone, $MgSO_4 7H_2O$, and K_2HPO_4. Sucrose was obtained from Difco; yeast extract and peptone from Himedia, while others were purchased from Merck. Fermentation media were prepared by mixing the materials with the composition of 2.5 % glucose, 2.5 % fructose, 5.0 % sucrose, 0.5 % yeast extract, 0.5 % peptone, 0.15 % $MgSO_4 7H_2O$, and 0.15 % K_2HPO_4.

Inoculum Preparation

Inocula were prepared by culturing each strain in a medium containing 2.5 % glucose, 2.5 % fructose, 5.0 % sucrose, 0.15 % $MgSO_4 7H_2O$, 0.15 % K_2HPO_4, 0.5 % yeast extract, and 0.5 % peptone in 250 ml Erlenmeyer flask with a working volume of 100 ml. Incubation was carried out on an orbital shaker at 100 rpm and temperature of 30°C, for 12 to 24 hours or until the cell density reached 10^8 cells ml^{-1}.

Ethanol Fermentation

Ethanol fermentation was performed in two steps. The first step was to elucidate the best strains combination in producing ethanol and the second was to describe the pattern of the changes in the concentration of ethanol, sugars and biomass during the fermentation progress. The first step was carried out using mixed cultures consisting of OUT7096/OUT7921, OUT7913/7921, and OUT7913/OUT7096. The second step was conducted using only the best cultures obtained from the first step.

The first step was started by mixing two selected *inocula* with ratios of 1:3, 1:1, and 3:1 into three of a one liter sterilized fermentation jars respectively and then 800 ml medium was added aseptically. The initial concentration of each strain is $7x10^7$cell ml^{-1}. Incubation was carried out at 30°C for 72 hours. The residual sugar and ethanol content were analyzed. The experiment was performed triplicates.

The second step was performed using only the best mixed culture obtained from the first step. During the incubation period, samples were collected every 4 hours and directly stored in a freezer for analysis of sugars and ethanol.

Ethanol yield ($Y_{p/s}$) is obtained by comparing the ethanol concentration to the concentration of sugars consumed during exponential phase. Mathematically, ethanol yield is calculated using:

$$Yp/s = \frac{p - po}{So - S} \quad \text{..... (1)}$$

P = final ethanol concentration;
P_0 = initial ethanol concentration
S = final substrate (sugars) concentration;
S_0 = initial substrate concentration

(Riadi, 2007)

Efficiency of sugar conversion to ethanol which expressing the ability of yeast to convert the available sugar into ethanol was calculated by equation:

$$SCE = \frac{alcohol\ content\ (\%\frac{W}{V})x100}{media\ sugar\ content\ (\%)x0.504} \quad \text{..... (2)}$$

SCE = sugar conversion efficiency

(*de* Mancilha *et al*., 1984[a])

Growth yield of biomass during fermentation progress was calculated using equation:

$$Yx/s = \frac{x - xo}{so - s} \quad \text{..... (3)}$$

X_0 and X = initial and final concentration of dried weight of biomass respectively;
S_0 and S = initial and final concentration of substrate respectively.

(Stanbury *et al*., 1995).

Analysis of sugars and ethanol was performed using an HPLC system (Knauer smartline RI detector 2300, Germany) using a column of Aminex HPX-87C 300 x 7.8 mm (Bio-Rad, USA) at 85°C. Injection volume is 20 µL and mobile phase is deionized water at flow rate of 0.6 ml min^{-1}.

Result and discussion

Determination of the best mixed cultures for sugar mixture fermentation

The results of first fermentation can be seen in Table 1. The table shows that both the highest ethanol concentration and the highest yield were produced by strain combination of OUT7921/OUT7913 and OUT7921/OUT7096 at ratio of 1:1. Single culture of strain OUT7906 as control also provided both a high ethanol concentration and yield but it was still lower than those produced by the both mixed culture of OUT7921/OUT7913 and OUT7921/OUT7096. The combination of OUT7913/OUT7096 was the only combination that produced lower both concentration and yield of ethanol compared with the controls. The excellence of OUT7921/OUT7913 mixed culture was in accordance with the results of our previous studies which showed that the OUT7921 strain excellence in the fermentation of

Table 1. Concentrations of ethanol and sugars after fermentation and ethanol yield

Strain Combination	strain ratio	ethanol (% v/v)	glucose (% w/v)	fructose (% w/v)	sucrose (% w/v)	sugar (% w/v)	consumed sugar (% w/v)	Yp/s ($ml.g^{-1}$)
OUT7921	1 : 3	4.59	0.04	0.10	0.33	0.47	9.53	0.48
+	1 : 1	**6.22**	0.09	0.14	0.08	0.31	9.69	0.64
OUT7096	3 : 1	4.11	0.02	0.08	0.18	0.28	9.72	0.42
OUT7921	1 : 3	4.49	0.04	0.08	0.44	0.56	9.44	0.48
+	1 : 1	**6.36**	0.00	0.06	0.15	0.21	9.79	0.65
OUT7913	3 : 1	3.88	0.02	0.10	0.25	0.36	9.64	0.40
OUT7913	1 : 3	3.06	0.06	0.11	0.01	0.18	9.83	0.31
+	1 : 1	5.58	0.01	0.10	0.02	0.13	9.87	0.57
OUT7096	3 : 1	2.98	0.03	0.03	0.01	0.07	9.93	0.30
Control								
OUT7921		5.97	0.01	0.09	0.26	0.36	9.64	0.62
OUT7913		5.63	0.01	0.07	0.08	0.15	9.85	0.57
OUT7096		6.09	0.00	0.12	0.33	0.45	9.55	0.64

sucrose and glucose-fructose mixture into ethanol, while the OUT7913 strain excellence in the fermentation of a mixture of glucose-fructose-sucrose (Jasman *et al*., 2012).

The mixed culture of OUT7913/ OUT7096 at ratio of 3:1 used most of sugar but it produced the least amount of ethanol. This result suggested that the combination of these two strains at this ratio was less efficient in converting sugar into ethanol compared with other cultures. This case may be caused by domination of one strain to another, so that, they could not work cooperatively. From Table 1, it can also be observed that the mixed culture which produced the highest concentration of ethanol was at ratio of 1:1. This may indicate that the two strains can work cooperatively and do not dominate each other. Effect of cell number ratio in the *inoculum* has been reported by Lee *et al*. (2013) in fermentation of papaya wine using *W. saturnus* and *S. cerevisiae*.

It can also be seen that the concentrations of glucose and sucrose residues after fermentation were almost always less than the concentration of fructose. It is suggested that the yeast prefers to consume glucose than fructose. This result is in accordance with the results of previous studies (Jasman *et al.*, 2012, Tronchoni *et al*., 2009; and Berthels *et al*., 2004).

Guillaume *et al.* (2007) assumed that differences in glucose and fructose consumption are due to the differences in transporting both compounds across cell plasma membranes. Analyses of the effect of *HXT* gene inactivation have shown that the *hexose* carriers Hxt1 to Hxt7 are the main transporters. These carriers classified into low-, intermediate-, and high-affinity transporters. Both high- and low-affinity transporters have a higher affinity for glucose than for fructose (Reifenberger *et al*., 1997). Berthels *et al* (2008) conclude that the glucose-fructose discrepancy in wine yeast strains correlates with the kinetic properties of *hexokinase*-mediated sugar *phosphorylation*.

Fermentation of sugar mixture with mixed culture of OUT7913/OU77921

Changes of sugars concentrations during the fermentation can be seen in Figure 1. Sucrose concentration decreased faster than the concentration of two other types of sugar. This is because the yeast producing *invertase* splitting sucrose molecules into glucose and fructose molecules. This membrane-bound enzyme breaks down sucrose into its *hexose* components during active transport of sucrose directly into the cell. This is an another uptake mechanism present in ethanolic yeasts (Rolz and de Leon, 2011).

Figure 1 shows that the decrease in glucose concentration was faster than that of fructose during the *log* phase. The decline of sucrose, glucose, or fructose is in line with the exponential increase of ethanol concentration. The results also agreed with the previous research which showed that common ethanol fermentation yeasts, strain of *S. Cerevisiae* consume sugar in mixtures of fermentable sugar in the order of sucrose, glucose, and fructose (Berthels *et al*., 2004; Meneses *et al*., 2002).

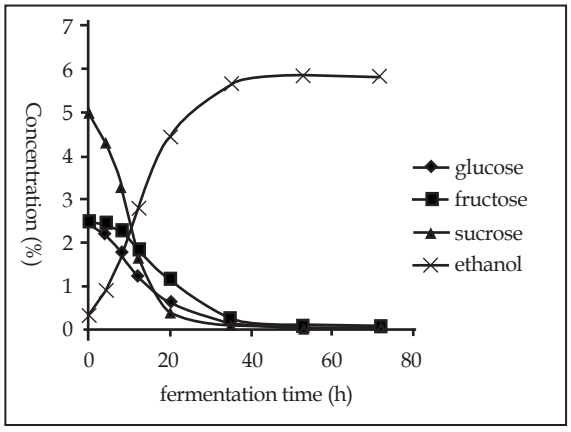

Figure 1. The changes of sugar and ethanol concentrations during fermentation at 30°C and initial pH 5,0.

Change in the concentration of ethanol during the fermentation was also shown in Figure 1. Ethanol concentration increase slowly until the 4th h and then it increase exponentially from the 4th h to 20th h. Further increase in the concentration of ethanol starts to slow down until it reaches a maximum, that was 5.86% v/v equal to 4.62% w/v, after another 37 h. Calculating the ethanol yield using equation (1) give $Y_{p/s}$ = 0.47 g.g^{-1} .

Using the equation (2), we obtained SCE = 93.25 which means that about 93.25% of substrate (sugars) can be converted to ethanol during the fermentation. This achievement is close to the value of 93.57% achieved by de Mancilha *et al.* (1984^{b}) who used *S. cerevisiae* IZ 1716 mut. on substrate of sweet sorghum juice.

The growth of yeast cells (biomass) during the fermentation can be seen on Figure 2.

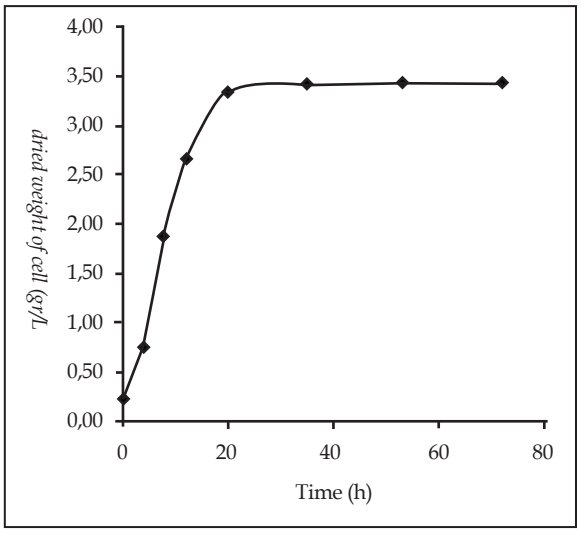

Figure 2. Biomass growth of yeast cells during fermentation of sugar mixtures at 30°C and initial pH 5.0

From the calculation, it was obtained $Y_{x/s}$ = 0.04 g.g^{-1} and μ = 0.12 h^{-1}. This value of specific growth was close to that of achieved by Pramanik *et al.* (2005) who used sucrose and a strain of *S. cerevisiae* isolated from toddy. These results indicated that mixed culture of yeast could work properly on substrate consisting of mixed sugars as well as single culture on substrate of single sugar.

In this study, the mixed culture at ratio of 1:1 can provide a satisfying result of fermentation on substrate consisting of sucrose, glucose, and fructose. Thus, the mixed culture is good for using in fermentation of natural substrates having sugar composition similar to those of used in this study such as sweet sorghum juice.

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